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In-Silico COMPARATIVE STUDY OF THREE (3) BIOACTIVE COMPOUNDS FROM METHANOL EXTRACTS OF *Combretum micranthum* LEAF, AND DIAZEPAM WITH $GABA_A$ RECEPTOR MOLECULE

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ABSTRACT
Stress affects monoamine neurotransmitter in the central nervous system such as GABA (a major inhibitory neurotransmitter in the brain). $GABA_A$ receptor is hetero-oligomeric Cl-channel that is elective blocked by the alkaloid, bicuculline and modulated by steroids, barbiturates and benzodiazepines. The anticonvulsant activity of Diazepam may be mediated by enhancement of inhibition involving gamma-aminobutyric acid (GABA). Combretum micranthum is one of the maximum effective medicinal plants of therapeutic importance. Thus this study is to examine the effect of Combretum micranthum methanol leaf extract on $GABA_A$ Receptor via In-Silico analysis. Combretum micranthum methanol leaf extract was found using GC-MS to contain bioactive compounds (3,5-dichlorophenylhydrazine, guanidine and aminooxyacetic acid) with GABAergic functions. And the popular docking programs PatchDock and AutoDockVina were then used to predict computationally binding modes of these compounds with $GABA_A$ receptor. The molecular docking analyses indicated highly and effectively interactions (binding energy in kcal/mol) between $GABA_A$ receptor and the Combretum micranthum compounds (ligands): 3,5-dichlorophenylhydrazine (-193.85 and-5.6), guanidine (-87.63 and -3.3) and aminooxyacetic acid (-85.3 and -3.2) for both PatchDock and AutoDock Vina respectively. Results shows that 3,5-dichlorophenylhydrazine has a close binding energy in kcal/mol to that of Diazepam (-200.68 and -6.1 respectively). Findings of the study shows that the interaction between Combretum micranthum compounds (3,5-dichlorophenylhydrazine, guanidine and aminooxyacetic acid) with $GABA_A$ receptor can be explore for the development of new therapeutics to manage mental disorders.
Keywords: Gamma-aminobutyric acid, Combretum micranthum , AutoDockVina, PatchDock

INTRODUCTION

Stress condition when unchecked, may lead to various mental and physical illnesses such as headaches, upset stomach, elevated blood pressure, chest pain, heart disease (Romero and Butler, 2007). These stress responses are governed by corticotrophin releasing hormone neurons. The activity of these neurons is largely controlled by robust Gamma- aminobutyric acid (GABA) mediated inhibition (Shalini *et al.,* 2018). GABA is the most widely distributed inhibitory neurotransmitter in the central nervous system, that influences mood conditions because it reduces high levels of the hormones adrenalin, noradrenalin and dopamine. GABA inhibits the cells from firing, diminishing the anxiety related messages from reaching the cortex (Glover *et al.,* 2010). All the functions of GABA are mediated through its specific receptors. GABA is formed within GABAergic axon terminals and released into the synapse, where it acts at one of two types of receptor: $GABA_A$ receptor which controls chloride entry into the cell, and $GABA_B$ receptor which increases potassium conductance, decreases calcium entry, and inhibits the presynaptic release of other neurotransmitters (Sieghart, 2006).

To lower down the effect of stress, different sedative medications are commonly recommended by clinicians to depress the activity of the central nervous system (causing a sense of relaxation, reduced anxiety and tension, sleepiness, and slowed breathing), such as the Benzodiazepines which include alprazolam (Xanax), clonazepam (Klonopin), diazepam (Valium), lorazepam (Ativan), triazolam (Halcion), temazepam (Restoril), and

chlordiazepoxide (Librium). Benzodiazepines produce their calming effects by activating and increases the effect of the neurotransmitter GABA, by binding to the benzodiazepine site on the $GABA_A$ receptor via the constituent chlorine atom (Riss *et al.,* 2008), which ultimately inhibit brain activity and thus slow and calm down the body ((Tanko *et al.,* 2009). However, the use of these drugs for long period is known to exhibit some severe side effects at physiological and psychological level such as slurred speech, impaired ability to walk around, poor judgment, slowed reflexes, low blood pressure, sexual dysfunction, memory loss. Hence, there is always a need of alternative therapy for stress management (Kinnersley and Turano*,* 2000). Various epidemiological studies have shown that a large number of herbs are known to help reducing and protecting against stress in animals (Fujisawa and Murakami, 2016). Additionally, these anti-stress herbs are safe for long term use (Pawar and Shivakumar, 2012; Khalil *et al.,* 2017).

Combretum micranthum commonly known as kinkeliba (health tree), is a shrub species often found in bushes and on hills in West Africa (Welch *et al.,* 2017), and also known locally as Géézà in (Hausa), Okan (Yoruba) and Nza otego (Igbo) in Nigeria, belongs to the family of Combretaceae (Burkill, 1985). It is a widely known ethnomedicinal plant used in West Africa for treating several conditions such as fatigue, liver ailments, headache, convalescence, blood disease, weight loss, cancer and sleep problems (Welch *et al.,* 2017). The present study aimed at evaluating the effect of *Combretum micranthum* methanol leaf extract on the central nervous system via $GABA_A$ Receptor by *In-Silico* analysis.

MATERIALS AND METHODS

MATERIALS

Collection and Identification of Plant

Fresh leaf of *Combretum micranthum* plant were obtained from Shira Local Government Area (N 11^O 27′ 29″ and E 10^O 2′ 48″) of Bauchi State in Nigeria. The plants were identified in the Herbarium Unit, Department of Biological Sciences, Bayero University, Kano. A voucher specimen number of BUKHAN 0272 was issued.

Chemicals, Reagents and Equipment

All the chemicals and reagents used for this work were of analytical grade and purchased from reputable chemical manufacturers, e.g. SIGMAALDRICH-FLUKA. The laboratory equipment used, were also of standard quality.

Ethical Clearance

Ethical clearance for the research was granted by the Bayero University, College of Health Sciences Research Ethics Committee with a reference number BUK/CHS/HREC/VII/62.

Preparation of the Extract

The leaves of *Combretum micranthum* plant were shade-dried, powdered to get a coarse powder and stored in a well closed container. The dried coarse powder was subjected to Microwave Assisted Extraction Method (HiNaRi Microwave Appliance: Model No.: MX 120BTC, 240V, 2450MHz, 1350Watts made in Korea). For Microwave-Assisted Extraction (MAE) of the *Combretum micranthum*, the powdered samples were mixed thoroughly with a suitable modifier (MeOH – H_2O in ratio of 4:1) as needed for the specific experiment. Sufficient time was allowed for the powder to absorb the modifier and get saturated. The saturated powder was then placed into the extraction vessel, and an appropriate amount of the extracting solvent was added. Different time of irradiation with the microwave extractor operating at an appropriate power level (850watts) is needed for MAE. The sample was treated in an intermittent way, i.e., irradiation–cooling–irradiation under microwave maintaining a particular ratio of irradiation and cooling time (5:5) (Waghmare *et al.,* 2015). The samples was filtered and concentrated to 1/10 volume (<40^oC). this was followed by acidifying it with 2M Tetraoxosulphate (vi) acid (H_2SO_4), then extracted with chloroform ($CHCl_3$ x3) giving two layers: $CHCl_3$ layer containing nonpolar compounds (Terpenoids) and the aqueous acidic layer containing highly polar compounds (flavonoids). The aqueous layer was then purified by basifying to pH 10 with sodium hydroxide (NaOH) and further extracted with chloroform-methanol (3:1) twice followed by extraction with chloroform. The aqueous basic layer was then concentrated and finally extracted with methanol (Harborne, 1989).

Phytochemical screening by GC-MS

About 0.5g of *Combretum micranthum* methanol leaf extract was dissolved in 95% methanol and filtered through microfilter 0.45 µm. the 2 µl of this solution was employed for GC/MS screening. GC-MS screening was carried out on a Shimadzu GCMS-QP2010Ultra system comprising a gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column Elite-1 fused silica capillary column (30×0.25 mm ID×1EM df, composed of 100% Dimethyl poly siloxane), helium (99.9%) was used as carrier gas at a Flow Control Mode: Pressure 100.0 kPa, Total Flow: 17.6 mL/min, Column Flow: 1.33 mL/min, Linear Velocity: 43.0 cm/sec, Purge Flow: 3.0 mL/min, Split Ratio:10.0, injector temperature 220°C; ion-source temperature 200°C.

The oven temperature was programmed from 100°C (isothermal for 2 min), with an increase of 10°C/min, to 200°C, then 5°C/min to 220°C, ending with a 9 min isothermal at 220°C. Mass spectra were taken at 70 eV, then the time required for sample chromatography was 20 minutes (Poaranthaman *et al.,* 2012). Phytocomponents were identified using MassHunter\Library\NIST14.L at Multi-User Science Research Laboratory Ahmed Bello University Zaria.

Docking analysis

The major active constituents of *Combretum micranthum* methanol leaves extracts identified and selected are as follows: 3,5-dichlorophenylhydrazine, guanidine and aminooxyacetic acid. These constituents were found to possess GABAergic properties according to literature claims (Matsuyama *et al.,* 1983, Bingham *et al.,* 2001, Heather and Eric*,* 2016). *In silico* docking analysis was performed using PatchDock (Schneidman *et al.,* 2005) and AutoDock Vina (Trott and Olson, 2010) to docked the selected compounds from the extract and diazepam (standard drug) to the target protein ($GABA_A$ receptor).

Retrieval of Protein and Ligand Structures from Database for Docking Analysis

The three dimensional structures of target protein namely $GABA_A$ receptor was retrieved from Research Collaboratory for Structural Bioinformatics (http://www.rcsb.org/pdb/home), with the PDB ID: 3D32 with resolution of 1.03Å, while the 3D structures of the various active constituents (ligands) of the extract were retrieved from PubChem chemical databases (https://pubchem.ncbi.nlm.nih.gov/compound): 3,5-dichlorophenylhydrazine (PubChem CID: 9321), guanidine (PubChem CID: 3520) and aminooxyacetic acid (PubChem CID: 286). For the standard compound, diazepam, it was downloaded from Drugbank website (https://www.drugbank.ca/drugs) with the ID: DB00829

Analysis of Physicochemical Properties

The physicochemical descriptions of all the compounds as well as ADME (absorption, distribution, metabolism, excretion/toxicity) properties were analyzed using SwissADME web tool (Daina *et al.,* 2017), developed and maintained by the Molecular Modeling Group of the Swiss Institute of Bioinformatics (SIB) (http://www.swissadme.ch/index.php).

Analysis of Target Protein Active Sites

Analysis of protein structures for binding site pockets is important and often the starting point to considered in the protein-ligand docking studies. In the present study, the CASTp server was used to identify the possible active binding sites for the ligand in the $GABA_A$ receptor. CASTp server predicted only one active site for the target protein (pocket 1). The area computed for the pocket 1 is 185.017 and volume 177.328. Pocket 1 comprises of 10 amino acid residues viz., ALA 4, LYS 7, LEU 1, AGR 10, SER 3, ASP 11, GLU 6, VAL 3, TYR 6 and PRO 3, as shown in the Fig. 1.

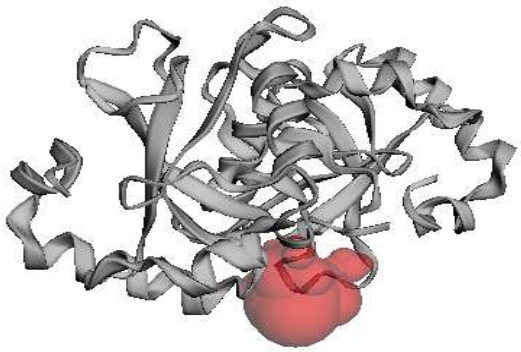

Fig. 1: Castp Result Showing Pocket in $GABA_A$ Receptor

Molecular Docking Studies with PatchDock

Molecular mechanics minimization of PDB protein structure and ligand structures were processed and carried out using Pymol (DeLano, 2002), and then employed for PatchDock analysis. PatchDock adopts geometry based molecular docking algorithm method to recognize the binding scores, binding residues and atomic contact energy of given ligands (Schneidman *et al.,* 2005). PDB format of both proteins and ligands were sent to PatchDock server for docking (http://bioinfo3d.cs.tau.ac.il/PatchDock/) setting clustering RMSD at 2.0 and complex type as default. The docking results were obtained through the user email address (providing the top 20 solutions in a table).

From these, top one solution (the docked protein-ligand complex) was selected and downloaded in program database (pdb) file format. Further, the binding site visualization was carried using PyMOL software (DeLano, 2002).

Molecular Docking Studies with AutoDockVina

Conformational analysis of docking was also performed using AutoDock Vina as stated by Rauf *et al.,* (2015). AutoDockVina is a standalone tool use in molecular docking. It is an improved version of AutoDock (Trott and Olson, 2010) which creates ligand poses by using genetic algorithm, Li *et al.,* (2010). Molecular mechanics minimization of PDB protein structure and ligand structures were optimized by using Merck Molecular Force Field (MMFF) and the semi-empirical Austin Model (AM1) method, both of which are implemented in Discovery studio visualizer (version 3.5, BIOVIA Software, www.3dsbiovia.com/product/collaborative-science/biovia-discovery-studio/) in order to remove all strain from the molecular structure. The fully optimized 3D structure without symmetry restrictions, were saved as SDF file through the file option on the Discovery studio visualizer 3.5. While using the protein preparation protocol, hydrogen atoms was added to the complex, after which water molecules are removed and the pH of the protein was set to almost neutral value. A sphere binding site with a nine Armstrong A° radius was defined around the bonded ligand to identify the binding site of the protein structure. The SDF files of the ligands were then imported into PyRx-virtual screening tool and they were used to dock the prepared receptors. The results of the best scored, binding energy and inhibition constants of all the ligands were reported on a table.

RESULTS AND DISCUSSION

Results

Phytochemical Composition by GC-MS Analysis

In the GC-MS analysis, 3 bioactive phytochemical compounds with GABAergic functions were identified and selected from extract: a) aminoxyacetic acid, constitutes of 0.91% peak area with retention time at 36.98, b) 3,5 –dichlorophenylhydrazine, constitutes of 3.73% peak area with retention time at 43.64 and c) guanidine, constitutes of 0.92% peak area with retention time at 88.09. The compounds were searched on various chemical databases for structure identification and found on PubChem Database with ID: CID286, CID9321 and CID3520 respectively (https://pubchem.ncbi.nlm.nih.gov/compound).

Analysis of Physicochemical Properties

In the present study, the physicochemical details and pharmacokinetic properties of identified compounds from the extract and standard drugs were studied to explore their binding mode with the $GABA_A$ receptor, as shown in Table 1 and Table 2 respectively.

Table 1: Physicochemical Properties of Compounds

Properties	Aminooxyacetic acid	3,5-dichlorophenylhydrazine	Guanidine	Diazepam
Formula	C2H5NO3	C6H6Cl2N2	CH5N3	C16H13ClN2O
Molecular weight	91.07 g/mol	177.03 g/mol	59.07 g/mol	284.74 g/mol
Num. heavy atoms	6	10	4	20
No. arom. heavy atoms	0	6	0	12
Fraction Csp3	0.50	0.00	0.00	0.12
Num. rotatable bonds	2	1	0	1
Num. H-bond acceptors	4	1	1	2
Num. H-bond donors	2	2	3	0
Molar Refractivity	17.29	43.67	15.93	87.95
TPSA	72.55 Å²	38.05 Å²	75.89 Å²	32.67 Å²

Table 2: Pharmacokinetic Properties of Compounds

Properties	Aminooxyacetic acid	3,5-dichlorophenylhydrazine	Guanidine	Diazepam
GI absorption	High	High	High	High
BBB permeant	No	Yes	No	Yes
P-gp substrate	No	No	No	No
CYP1A2 inhibitor	No	No	No	Yes
CYP2C19 inhibitor	No	No	No	Yes
CYP2C9 inhibitor	No	No	No	Yes
CYP2D6 inhibitor	No	No	No	Yes
CYP3A4 inhibitor	No	No	No	Yes
Log K_p (skin permeation)	-9.23 cm/s	-5.66 cm/s	-7.61 cm/s	-5.91 cm/s

Molecular Docking Analysis

The ligands (diazepam, guanidine, 3,5 -dichlorophenylhydrazine and aminoxyacetic acid) in the present study were docked to the target receptor ($GABA_A$ Receptor), using an automated molecular docking server PatchDock in order to find out how the chemical compounds interact with the target proteins structure based on the negative binding affinity values (Mashiach *et al.,* 2010) as shown in Table 3. Docking studies yielded crucial information concerning the orientation of the ligands in the binding pocket of the target protein. The minimum binding energy indicated that the $GABA_A$ Receptor was successfully docked with the ligands (diazepam, guanidine, 3,4 -dichlorophenylhydrazine and aminoxyacetic acid).

Further confirmation of the docking was also carried out using Autodock Vina tool and results confirmed the PatchDock results, showing successful binding of the ligands (diazepam, guanidine, 3,4 -dichlorophenylhydrazine and aminoxyacetic acid) with the $GABA_A$ Receptor target protein Table 4. The best proposed structural hypothesis of how the ligands (diazepam, guanidine, 3,4 -dichlorophenylhydrazine and aminoxyacetic acid) from the present study interacted with the target receptor ($GABA_A$ Receptor) as provided by the results from PatchDock and Autodock Vina, are given in figure 1 and figure 2 respectively. The stability of the compounds, which is based on the binding energy is in the order: diazepam>3,4 -dichlorophenylhydrazine >aminooxyacetic acid>guanidine.

Table 3: Binding Site Analyses of Best Ligands and Receptor Interaction by PatchDock

Complex	Score	Attractive Vdw	Repulsive Vdw	Atom Contact Energy (kcal/mol)
$Gaba_A$-3,4 -dichlorophenylhydrazine	2600	-9.38	-1.11	-7.95
$Gaba_A$-Guanidine	1462	-4.37	0.42	-4.72
$Gaba_A$-Aminooxyacetate	1900	-5.96	0.50	-4.76
$Gaba_A$-Diazepam	3364	-17.15	-8.51	-8.87

Table 4: Binding Site Analyses of Best Ligands and Receptor Interaction by AutoDock Vina

Complex	Binding Affinity (kcal/mol)	RMSD/ub	RMSD/lb	Protein Residue	Hydrogen Bonding	SASA
$Gaba_A$-3,4 -dichlorophenylhydrazine	-5.6	3.50	2.36	GLU	1	20.0
$Gaba_A$-Guanidine	-3.3	2.01	0.15	LEU, ASP, PRO	3	22.5
$Gaba_A$-Aminooxyacetate	-3.2	1.60	1.63	ARG, SER,PRO	4	22.5
$Gaba_A$-Diazepam	-6.1	3.81	2.57	PHE	1	17.5

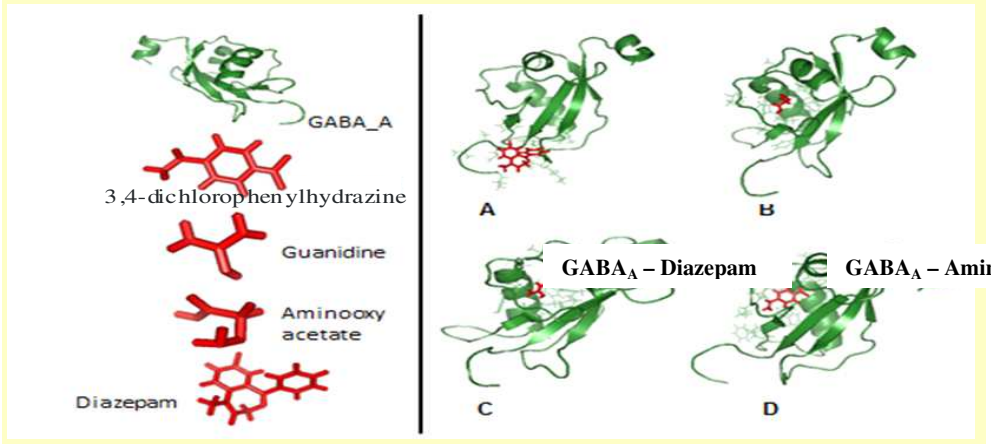

Figure 1: The Best Docked Result of Receptor – Ligand Complexes: Molecular docking stimulation done by PatchDock, 3D structure Generated by Pymo

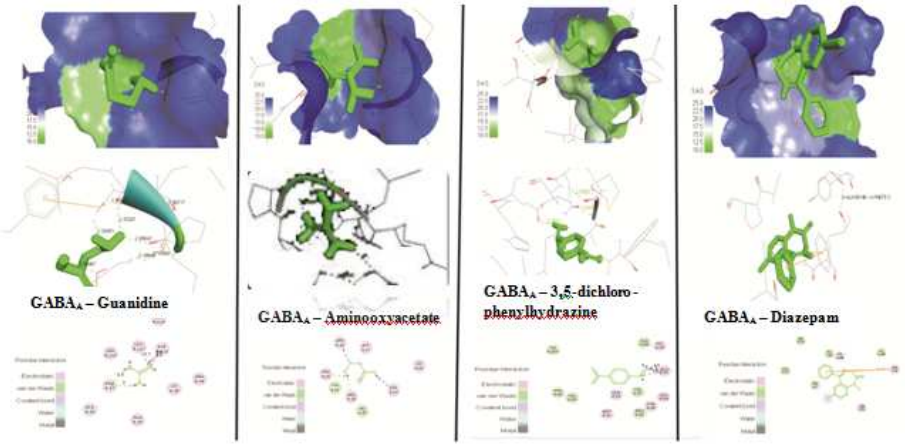

Figure 2: The Molecular Interaction between Receptor and Ligand: Molecular docking stimulation done by AutoDockVina, 3D structure Generated by Discovery Studio Visualizer.

DISCUSSION

The phytochemicals were evaluated to identify their potential capacity to act as a drug or lead compound. Prediction of interaction energies between ligand and receptor has been a major challenge for molecular docking. The docking tools used predicts binding property of ligands based on their structure, functional groups and molecular properties such as molecular weight (MW), number of Hydrogen Bond Donors (HBD), number of Hydrogen Bond Acceptors (HBA) and Solvent Accessibility Surface Area (SASA). The stability of the docked complexes increases with decrease in binding energy value. While comparing the results obtained for all the selected compounds of the extract, it is prominent that all of them show very good stability when docked with $GABA_A$ receptor. It is noted that the energy value is comparatively less for the model structure of 3,5-dichlorophenylhydrazine(-193.85 kcal/mol) than guanidine(-87.63 kcal/mol) and aminooyacetate (-85.33 kcal/mol) in both PatchDock, hence conforming highest stability (Schneidman *et al.,* 2005). These results shows that the binding value of the selected compounds from the *Combretum micrathum* methanol extract with $GABA_A$ receptor was very good for a compound to show such binding conformation with that protein, as with diazepam (-200.68) Table 3.

The confirmation of docking scoring was further carried out by AutoDock Vina, and the results also supported PatchDock output. Moreover, 3,5-dichlorophenylhydrazine determines to be the most prominent compound of the extract giving the least binding affinity values with $GABA_A$ receptor of -5.6 kcal/mol Table 5 (Li *et al.,* 2010). Also the successful binding of these compounds to the target proteins pockets Fig. 2 and Fig. 3, can be considered as potential modulators of $GABA_A$ receptor

Diazepam used in the study is to illustrate the active property of drug in the central nervous system (Vogel, 2008). Benzodiazepines (BZD), used as classic anxiolytics drug, induced its activities in the brain because of its regulatory effect exerted on $GABA_A$ receptor (Tanko *et al.,* 2009). BZDs interact with their recognition site in an agonist-like manner by directly activating $GABA_A$ receptors via positive modulation, thereby resulting in downstream conformational changes that stabilize the active state(s) of the receptor (Downing *et al.,* 2005; Rusch and Forman, 2005). In the present study analysis, the natural compounds which exhibited almost similar binding affinity like diazepam, was suggested same binding to $GABA_A$ receptor. Further the conformation with least binding affinity was considered to be the most suitable docking pose. It was observed that out of all the natural compounds, 3,5-dichlorophenylhydrazine showed the binding affinity close to diazepam (−193.85 and −5.6 kcal/mol respectively), therefore could be use as substitute for the drug, as in Table 3 and Table 4. While the other two ligands: aminooxyacetic acid and guanidine both showed relatively low affinity (about 50% as compared to diazepam) towards the GABAa receptor and might not be considered further.

CONCLUSION

In the present *in-silico* investigation, it was elucidated that the compounds (3,5-dichlorophenylhydrazine, guanidine and aminooxyacetate) identified from the plant (*Combretum micranthum)* methanol leaf extract acted as potential neuromodulators for the target protein, GABAa receptor. Analysis of ligand binding interaction with the target proteins can be useful for new preventive and therapeutic drug for neurodegenerative disorders.

Based on the molecular drugs docking and binding affinities of the target protein with the compounds (3,5-dichlorophenylhydrazine, guanidine and aminooxyacetate) and their physicochemical detailing, it was found that the plant (*Combretum micranthum*) extract, have high possibilities to serve as a substitute for the synthetic anti-stress drugs and can be better used for the development of new therapeutics to modulate GABA activities in the brain.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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